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(54) Title: **INHIBITORS OF RETROVIRAL PROTEASE AS INDUCERS OF REVERSIBLE INSULIN RESISTANCE *IN VITRO*
AND *IN VIVO***

(57) Abstract: This invention provides novel assays for measuring the metabolic side-effects of antiretroviral protease inhibitors on the Glut4 glucose transporter. The invention also provides improved methods for developing antiretroviral protease drugs, particularly those used to fight HIV infection. The invention further provides novel models of insulin-resistant glucose transport disease states.

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**Inhibitors of Retroviral Protease as Inducers of
Reversible Insulin Resistance In Vitro and In Vivo**

5 This application claims priority to U.S. Provisional
Application No. 60/198,524, filed April 19, 2000, the
entirety of which is incorporated by reference herein.

10 Pursuant to 35 U.S.C. §202(c), it is acknowledged
that the U.S. Government has certain rights in the
invention described herein, which was made in part with
funds from the National Institutes of Health, Grant No.
DK38495.

15 **Field of the Invention**

 This invention relates to the field of cellular
biology and disease states, including HIV infection and
diabetes. Specifically, this invention provides novel
assays for the effects of antiretroviral protease
20 inhibitors on the Glut4 glucose transporter and a novel
in vivo model of insulin-resistant glucose transport
disease states.

Background of the Invention

25 Several publications are referenced in this
application in order to more fully describe the state of
the art to which this invention pertains. Each of these
publications is incorporated by reference herein.

 The development of new targets for therapeutic
30 agents for the treatment of HIV infections, as well as
powerful combinations of those therapeutic agents has led
to what is now commonly referred to as HAART (for highly-
active antiretroviral therapies). Among the cornerstones
of the therapeutic components of these HAART approaches

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5 are the antiretroviral protease inhibitors which have
been developed. As part of combination therapies, HIV
protease inhibitors play a critical role in suppressing
viral titers and increasing CD4+ lymphocyte counts, which
can result in significantly reduced mortality among HIV
10 patients.

The human immunodeficiency virus (HIV) genome
encodes an aspartyl protease that is required to process
its viral precursor polyproteins to their mature forms.
This protease activity is essential for the proper
15 formation of infectious HIV virions. The development of
a class of specific agents that target the HIV protease
was an extraordinary advance in the treatment of HIV
infection.

Despite the rapid progress in the treatment of the
20 disease, and the improved prognosis for those infected,
it now appears clear that the use of protease inhibitors
is associated with potentially serious side effects. In
1997, the FDA issued a Public Health Advisory to
healthcare professionals warning that use of protease
25 inhibitors was associated with increases in blood sugar
and diabetes. Eighty-three cases had been reported, of
which twenty seven required hospitalization. Diabetes
cases were associated with use of several available
protease inhibitors, including indinavir, nelfinavir,
30 ritonavir, and saquinavir.

Although the mechanisms have not been elucidated, it
is clear that protease inhibitor use is linked in some
manner to a syndrome of alarming metabolic abnormalities
characterized by hypertriglyceridemia,
35 hypercholesterolemia, peripheral fat wasting, central
adiposity and hyperglycemia and insulin resistance. The

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5 etiology of this metabolic syndrome associated with
protease inhibitor use currently remains unknown, but its
features are similar to those present in the insulin-
resistant state commonly referred to as Syndrome X.
Common symptoms of the syndrome include body fat changes
10 including: enlarged dorsicervical fat pads (known as
"buffalo hump"); abundance of benign lipomas; deposition
/ accumulation of fat in the abdomen or viscera (Crix
belly); breast hypertrophy; and a characteristic loss of
fatty tissue from the face and extremities. Collectively
15 these body fat changes are referred to as lipoatrophy, or
more commonly, lipodystrophy. Hyperlipidemia and insulin
resistance appear to occur with high prevalence among
patients using protease inhibitors, such that increased
risk of premature cardiovascular disease and diabetes are
20 relevant issues. The prevalence of lipodystrophy has
been reported to be as high as 83% according to one
study. Other studies suggested that some of the symptoms
may appear to varying degrees in patients not treated
with protease inhibitors, or that symptoms vary depending
25 upon which protease inhibitor was used for treatment.
Yet other studies revealed possible differences in
patients based on age, gender, length of infection and
other factors such as change in weight and hemophilia.

Thus, sustained treatment with the currently
30 available antiretroviral protease inhibitors results in
at least some, or all, of these metabolic disturbances,
particularly those associated with hyperlipidemia and
insulin resistance. The long-term, or even near-term,
risk to the health of patients, while less than the
35 imminent risk of uncontrolled viral growth, is

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5 substantial. The ability to design drugs lacking the
undesirable side-effects is widely recognized as a need
in the art and would be of great significance in
combating HIV and other retroviral diseases.
Furthermore, a more detailed understanding of the
10 molecular mechanisms which lead to this metabolic
disorder would contribute to the development of novel
experimental or in vivo models related to the generalized
problem of insulin resistance.

15 **Summary of the Invention**

It has now been discovered in accordance with the
present invention that anti-HIV drugs of the protease
inhibitor class, including, for example, indinavir,
ritonavir and amprenavir, are selective inhibitors of the
20 Glut4 glucose transporter. This discovery enables the
development of assays for use in screening of protease
inhibitors for this unwanted and undesirable side-effect;
in addition, improved procedures for the rational design
and testing of antiretroviral protease inhibitors are
25 enabled by this novel method.

The present invention provides novel assays for
determining the effect of protease inhibitors on glucose
transport activity. The invention also provides methods
for screening antiretroviral protease inhibitor drugs for
30 side-effects related to glucose transport in the presence
or absence of insulin stimulation. In its most basic and
general form, the assay method comprises the steps of:
providing glucose transporters of interest in a membrane
system; incubating the glucose transporters with a
35 measurable form of glucose or a glucose analog; adding to

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5 the incubation mixture a antiretroviral protease
inhibitor being screened; optionally, stimulating the
glucose uptake of the cells with insulin; and quantifying
the glucose transport by determining the difference in
10 the amount of glucose uptake in the presence and in the
absence of the protease inhibitor being screened, and
optionally, in the presence or absence of insulin, or
other glucose transport-altering substances. The
invention provides, in one embodiment, that the glucose
transporter is a Glut4 isoform. In one basic embodiment
15 the membrane system comprises cells, for example
adipocytes, producing a glucose transporter. The
invention provides a wide variety of cells for use with
the methods.

Also provided in accordance with the present
20 invention is a method for testing a wide variety of
antiretroviral protease inhibitors for side-effects on
glucose transport. Included among the protease
inhibitors and their derivatives to be tested by the
method above are various compounds and families of
25 compounds, many of which are already established as
inhibitors of aspartyl proteinases.

The invention also provides for a variety of glucose
compounds to be used as the detectable glucose. Glucose
compounds include glucose, and its analogs, including
30 transportable analogs of glucose.

The invention also provides methods for measuring
the side-effects of protease inhibitors on glucose
transport in cell-free systems. The advantages of cell-
free systems are well known in the art. In the cell-free
35 methods of the invention, the membrane system selected

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5 comprises a naturally-derived membrane from cells in certain embodiments, while in others, a noncellular system, for example, artificial membranes or vesicles, is used with the glucose transporter isoforms.

10 Also provided for use in the methods of the present invention when the membrane system selected is cells, are cells containing one or more expressible nucleic acids encoding one or more glucose transporters. The invention provides that the cellular expression of the glucose transporter is a stable characteristic of the cell line
15 in certain embodiments. In other embodiments, the expression of the glucose transporter is transient. The expressible nucleic acids encode one or more homologous or heterologous glucose transporters of interest. In one embodiment, the cells have a substantially negligible
20 basal level of glucose transport, other than the glucose transport provided by the heterologous glucose transporter.

Also provided in accordance with the present invention are kits for testing protease inhibitors for
25 side-effects involving glucose transport. The utility of such kits is well established. The kits of this invention can include a cell line complete with heterologous glucose transport expression capability, or another membrane system with glucose transporter
30 isoforms, and a detectable glucose compound, such as glucose or a glucose analog. Further included are standards for insulin stimulation, and protease inhibitors for standardizing the inhibition assays.

The invention further provides a method for the
35 rational design of new antiretroviral treatments. The

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5 method provides that a candidate drug or lead compound be
subjected, in the early stages of the drug development,
to assays to measure effects on glucose transport. The
assay method comprises the steps of identifying a
therapeutic test compound; testing the compound by:
10 determining that it inhibits a retroviral aspartyl
protease; using the compound as a protease inhibitor in
the screening method described above; assessing the
compound for inhibition of glucose transport; and
selecting those compounds which inhibit the aspartyl
15 protease and which do not inhibit glucose transport.
Candidates of greatest interest for further development
are those which maximally inhibit the retroviral protease
but do not substantially alter the glucose transport
activity in either the presence or absence of insulin
20 stimulation.

Further provided in the invention are methods of
cell-free methods of rapidly screening protease
inhibitors for specific molecular interactions with
glucose transporters. Such specific molecular
25 interactions are an indication of potential inhibition or
undesirable side-effects of protease inhibitors. These
methods provide for labeled glucose transporters and or
labeled protease inhibitors. The invention provides for
measuring the specific molecular interactions between
30 either a membrane-associated or solubilized glucose
transporter and a protease inhibitor.

Also provided in accordance with the present
invention is a method of screening factors, compounds or
conditions which alter reversible insulin-resistant
35 glucose transport. Compounds identified by such a method

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5 would be excellent candidates for treating disease
conditions comprising insulin resistance. The method
comprises the steps of: providing a cell line producing
one or more glucose transporters; incubating the cells
with a detectable glucose or glucose analog in the
10 presence of an inhibitor known to specifically inhibit
the glucose transporter isoform of interest; including a
compound or condition whose effects on reversing the
inhibition are to be tested; and quantifying the reversal
of glucose transport inhibition. Also provided are such
15 assays in either the presence or absence of insulin or
other glucose transport-altering substances.

Other features and advantages of the present
invention will be understood by reference to the detailed
description of the invention and examples that follow.

20

Brief Description of the Drawings

Figure 1. The effect of HIV protease inhibitors on
glucose uptake in 3T3-L1 adipocytes.

25 Figure 1A. Cells were incubated at 37 °C in the
presence of indinavir sulfate, added to final
concentrations of 10, 20, 50, or 100 μ M (as indicated on
the X-axis), for 6 min prior to the glucose uptake assay.
[³H]-2-deoxyglucose uptake was measured for 6 min under
basal and insulin-stimulated conditions (incubation with
30 1 μ M insulin for 20 min prior to assay). Results from
three experiments were normalized to the value obtained
from insulin-stimulated control cells and are shown as
the mean \pm S.E. (n = 6).

35 Figure 1B. 3T3-L1 adipocytes were treated with
either no protease inhibitor (Control), or with

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5 ritonavir, indinavir, or amprenavir, at 50 μ M. [3 H]-2-deoxyglucose uptake was measured as described above. Results were normalized to the value obtained from insulin-stimulated control cells and are shown as the mean \pm S.E. ($n = 3$).

10 **Figure 2. Indinavir does not affect insulin signaling or glucose transporter translocation.**

Figure 2A. Mature 3T3-L1 adipocytes were incubated in serum-free DMEM for 4 h with or without 100 μ M indinavir. Cells were further incubated for 20 min with or without 1 μ M insulin, and subcellular fractions were subsequently isolate. Plasma membrane (PM), low density microsomal (LDM), and cytosolic (CYT) fractions were subjected to immunoblot analysis using anti-phosphotyrosine antibodies. The positions of the tyrosine-phosphorylated insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) are indicated by arrows.

20 Figure 2B. Whole cell lysates from 3T3-L1 adipocytes from samples treated as described above were subjected to immunoblot analysis using anti-phospho Akt antibodies, which recognize Akt phosphorylated on threonine 308 and serine 473.

25 Figure 2C. Relative Glut1 and Glut4 transporter isoform contents in the PM and LDM subcellular fractions from samples as described above were visualized by immunoblot using isoform-specific polyclonal antibodies.

30 **Figure 3. Inhibition of glucose uptake in *X. laevis* oocytes by HIV protease inhibitors.**

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5 Figure 3A. *Xenopus* oocytes heterologously
expressing either Glut1 or Glut4 isoforms were used for
[³H]-2-deoxyglucose uptake measurement in Barth's Saline
at 22 °C for 30 min. Immediately prior to the uptake
measurement, indinavir sulfate (at the final
10 concentrations (in μ M increments) indicated on the X-
axis) was added to the assay mixture. Plotted are the
mean uptake from 15-20 oocytes \pm S.E.. [* indicates $p <$
0.01 compared with control (ANOVA with Fischer's PLSD
posthoc analysis).]

15 Figure 3B. Glucose uptake in *X. laevis* oocytes as
described above. Control assays are compared to those in
which either ritonavir, indinavir or amprenavir at 50 μ M
were added to the assay mixtures immediately prior to the
uptake measurements. The data are normalized to the
20 uptake from the control oocytes which were not exposed to
protease inhibitor. [* indicates $p < 0.0001$ compared
with control (ANOVA with Fischer's PLSD posthoc
analysis).]

25 Figure 4. Effect of acute indinavir administration
on glucose tolerance in rats.

Figure 4A. Following a 12 hour overnight fast, male
Wistar rats weighing 250 - 400 grams were given a single
intraperitoneal dose of 50% dextrose (2 gm/kg) together
30 with indinavir (10 mg/kg) or water. From samples of
peripheral venous blood, plasma glucose levels were
measured using a Glucometer Elite glucometer. Each data
point represents the mean \pm SEM values from 8-9 rats.

Figure 4B: A jugular venous catheter was inserted,
35 at least 4 days prior to each experiment, into 200 - 300

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5 gm male Wistar rats under methohexital anesthesia.
Following a 12 hour fast, rats were injected with
indinavir (15 mg/kg in normal saline) or saline through
the venous catheter 5 minutes prior to intraperitoneal
injection of 50% Dextrose (2 gm/kg). Plasma glucose
10 levels were measured from blood sampled from the venous
catheters. Values represent the mean \pm SEM from 4-5
rats. [* indicates $P < 0.05$.]

Figure 5. Effect of acute intravenous
15 administration of indinavir on peripheral insulin
sensitivity in rats.

Jugular vein and carotid artery catheters were
inserted, at least 4 days prior to each experiment, into
200 - 400 gm male Wistar rats under methohexital
20 anesthesia. Following an overnight fast, rats were
infused with insulin (40 mU/kg/min) and 50% dextrose
through the venous catheter. Blood was sampled every 5-
10 minutes through the arterial catheter and serum
glucose concentrations were determined. The glucose
25 infusion rate (GIR) space was adjusted to maintain
glucose levels of 100-110 mg/dl. Solid arrows represent
the start of a continuous infusion of water containing
indinavir through the intravenous line. Open arrows
represent the discontinuation of the indinavir infusion.

30 Figure 5A: Control Experiment (no indinavir), Figure
5B: 0.3 mg/kg/min indinavir, Figure 5C: 0.5 mg/kg/min
indinavir.

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5 Figure 6. Reduction in peripheral insulin
sensitivity induced by acute intravenous indinavir
administration in rats.

Male Wistar rats weighing 200-300 gm were
catheterized as described in Figure 5. Following an
10 overnight fast, a continuous infusion of water containing
0, 0.3 or 0.5 mg/kg/min indinavir was started through the
venous catheter. After 30 minutes, insulin (40
mU/kg/min) and 50% dextrose were added to the intravenous
infusion. Blood was sampled through the arterial line
15 every 5 min and the dextrose infusion rate was adjusted
to maintain plasma glucose levels between 105-115 mg/dL.
GIR represents the mean glucose infusion rate during the
final 30 minutes of each 2 hour clamp experiment.
Results represent the mean \pm SEM from 3-5 rats per
20 group. [* indicates $P < 0.5$]

Detailed Description of the Invention

1. Definitions

25 Various terms relating to the biological molecules
of the present invention are used hereinabove and also
throughout the specifications and claims. Certain
aspects of the present invention employ conventional
30 molecular biology, microbiology, and recombinant DNA
techniques that are well known in the art. See, e.g.,
Sambrook et al., "Molecular Cloning: A Laboratory Manual
(1989); or "Current Protocols in Molecular Biology", eds.
Frederick M. Ausubel et al., John Wiley & Sons, 1999.

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5 If appearing herein, the following terms have the definitions set out below.

With reference to nucleic acid molecules, the term "isolated nucleic acid" is sometimes used. This term,
10 when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous (in the 5' and 3' directions) in the naturally occurring genome of the organism from which it was derived. For example, the "isolated nucleic acid" may
15 comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a procaryote or eucaryote. An "isolated nucleic acid molecule" may also comprise a cDNA molecule.

With respect to RNA molecules, the term "isolated nucleic acid" primarily refers to an RNA molecule encoded
20 by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from RNA molecules with which it would be associated in its natural state (i.e.,
25 in cells or tissues), such that it exists in a "substantially pure" form (the term "substantially pure" is defined below).

With respect to proteins or peptides, the term "isolated protein (or peptide)" or "isolated and purified
30 protein (or peptide)" is sometimes used herein. This term refers primarily to a protein produced by expression of an isolated nucleic acid molecule of the invention. Alternatively, this term may refer to a protein which has been sufficiently separated from other proteins with

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5 which it would naturally be associated, so as to exist in
 "substantially pure" form.

 The term "substantially the same" refers to nucleic
acid or amino acid sequences having sequence variation
that do not materially affect the nature of the protein
10 (i.e. the structure, stability characteristics, substrate
specificity and/or biological activity of the protein).
With particular reference to nucleic acid sequences, the
term "substantially the same" is intended to refer to the
coding region and to conserved sequences governing
15 expression, and refers primarily to degenerate codons
encoding the same amino acid, or alternate codons
encoding conservatively substituted amino acids in the
encoded polypeptide. With reference to amino acid
sequences, the term "substantially the same" refers
20 generally to conservative substitutions and/or variations
in regions of the polypeptide not involved in the
determination of structure or function.

 A "coding sequence" or "coding region" refers to a
nucleic acid molecule having sequence information
25 necessary to produce a gene product, when the sequence is
expressed.

 As used herein a "heterologous" protein is a protein
produced by an organism that is not the wild-type source
of that protein. For example, *Xenopus* cells which have
30 been genetically modified to produce a glucose
transporter from rat nucleic acids are producing a
heterologous glucose transporter. A heterologous protein
is non native or exogenous to the organism producing it.

 A "heterologous" region of a nucleic acid construct
35 is an identifiable segment (or segments) of the nucleic

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5 acid molecule within a larger molecule that is not found
in association with the larger molecule in nature. Thus,
when the heterologous region encodes a mammalian gene,
the gene will usually be flanked by DNA that does not
flank the mammalian genomic DNA in the genome of the
10 source organism. In another example, coding sequence is a
construct where the coding sequence itself is not found
in nature (e.g., a cDNA where the genomic coding sequence
contains introns, or synthetic sequences having codons
different than the native gene). Allelic variations or
15 naturally-occurring mutational events do not give rise to
a heterologous region of DNA as defined herein.

A cell has been "transformed" or "transfected" by
exogenous or heterologous DNA when such DNA has been
introduced inside the cell. The transforming DNA may or
20 may not be integrated (covalently linked) into the genome
of the cell. In prokaryotes, yeast, and mammalian cells
for example, the transforming DNA may be maintained on an
episomal element such as a plasmid. With respect to
eukaryotic cells, a stably transformed cell is one in
25 which the transforming DNA has become integrated into a
chromosome so that it is inherited by daughter cells
through chromosome replication. This stability is
demonstrated by the ability of the eukaryotic cell to
establish cell lines or clones comprised of a population
30 of daughter cells containing the transforming DNA. A
"clone" is a population of cells derived from a single
cell or common ancestor by mitosis. A "cell line" is a
clone of a primary cell that is capable of stable growth
in vitro for many generations.

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Where used herein "retroviral protease" refers to the major aspartyl protease common to retroviruses, such as HIV. The retroviral proteases are known to be important to the life-cycle of retroviruses, and have become an important therapeutic target for new drugs.

10

The term "protease inhibitor" refers to a compound which inhibits the proteolytic action of a proteolytic enzyme. The inhibition may be through a variety of physical and chemical mechanisms, depending the type and structure of the active site of the protease.

15

The term "antiretroviral" generally refers to a class of drugs used to therapeutically treat infections with retroviruses. The term is also sometimes used herein to refer the properties of a protease inhibitor; i.e. the "antiretroviral properties" of a particular inhibitor are those properties which make it particularly inhibitory to the life cycle of a retrovirus.

20

The term "antiretroviral protease inhibitor" refers specifically to that subclass of the larger group of protease inhibitors which have activity against retroviral proteases, particularly the proteases of medically significant retroviruses of man and animals. The term as used herein sometimes refers more specifically to a group of protease inhibitors which inhibit the aspartyl protease of HIV.

25

30

The term "glucose analog" refers to derivatives of the glucose molecule. Glucose analogs include naturally occurring molecules, and synthetic derivatives. In general the term includes both transportable and nontransportable analogs. A glucose analog may include

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5 labels, as with fluorescent glucose analogs, or isotopically labeled glucose analogs.

Where used herein the term "glucose compound" refers to glucose itself, or any glucose analog. The term is often used in the sense of a "detectable glucose
10 compound" or a "detectable glucose" which includes any glucose or glucose analog which can be detected by chemical, enzymatic, physical or other means of detection.

The term "glucose transport-altering substance" as
15 used herein includes hormones, such as insulin, synthetic hormones, hormone analogs, drugs which alter glucose transport, inhibitors, or any compound known to alter the transport of glucose. The term "alteration" or
"altering" of glucose transport includes both stimulating
20 and inhibiting alterations, or increases or decreases in glucose transport across a membrane.

Where used herein, the term "molecular interactions" or "physical interaction" broadly refers to the relationship between two molecules, and includes, for
25 example, interactions such as hydrophobic interactions, ionic interactions, hydrophilic interactions, such as water structure, Van der Waal's interactions, covalent interactions. Also included within the meaning of the term are more complex "biological" interactions such as
30 binding site interactions, which typically represent the collective sum of numerous smaller physical and chemical interactions of one or more types, including hydrogen bonding and other transient or statistical interactions of atoms and molecules.

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5

2. Description

The undesired side-effects of anti-HIV protease inhibitors have come to be recognized as a syndrome of metabolic disorders often referred to collectively as lipodystrophy. In the present invention, a selective inhibitory action of these protease inhibitors on the Glut4 glucose transporter isoform, but not on the Glut1 isoform, is an important and novel finding. This finding leads to several useful applications in the screening and rational design of antiretroviral protease inhibitors. In addition, the discovery of selective, reversible, insulin-resistant glucose transport allows for methods to screen drug candidates and factors affecting such transport. Such drugs are needed for the treatment of insulin-resistant disease conditions.

In accordance with the present invention, a method is provided for the screening of protease inhibitors for metabolic side-effects. The method comprises the following steps: providing one or more glucose transporter isoforms in a membrane system; incubating the glucose transporters with a detectable glucose or glucose analog; adding a protease inhibitor of interest; determining activity of the glucose transporter by measuring an amount of glucose transported; and quantifying the metabolic side-effects of the protease inhibitor as a function of the activity of the glucose transporter in the presence of the protease inhibitor compared to that in the absence of the protease inhibitor.

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5 In one embodiment, the membrane system comprises intact cells. The cells may be primary cells or cell cultures. The cells may originate from different tissues known to possess glucose transporter activity. Such tissues include, but are not limited to, adipocytes, kidney cells, cardiac cells, skeletal muscle cells, liver
10 cells, neuronal cells and brain cells. In another embodiment tissues are from rapidly dividing cells, cell-lines or cell populations such as tumor cells, ascites cells, cancerous cells, or transformed cells.

15 In another embodiment cells are genetically modified or engineered for expression or over-expression of a gene or cDNA. In a preferred embodiment the gene or cDNA encode glucose transporter isoform from a gene or cDNA encoding a glucose transporter.

20 In another embodiment, cells which have a very low amount of basal glucose transport, such as *Xenopus* oocytes, are used to transiently express a glucose transporter protein of interest from a nucleic acid encoding the glucose transporter protein.

25 In one embodiment, the invention provides cells capable of expressing a gene, cDNA, or mRNA encoding one or more glucose transporters. The glucose transporter expressed comprises one or more specific glucose transporter isoforms of interest. In one embodiment, the
30 glucose transporters are expressed transiently; in another embodiment they are expressed in stable fashion. In a preferred embodiment, the glucose transporter is heterologous to the cell line and glucose transport through the heterologous transporter is readily
35 distinguished from the cell's basal level of glucose transport.

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5 The expression of the glucose transporter may be in
Xenopus laevis oocytes or other cells such as yeast cells
(e.g. *Saccharomyces cerevisiae*), insect cells, mouse
cells, rat cells, rabbit cells, chimpanzee cells, human
cells, or other eukaryotic cells capable of expressing a
10 homologous or heterologous glucose transporter.
Prokaryotic cells are also contemplated for use with the
methods of this invention. Proper expression of
eukaryotic transport proteins in prokaryotes is known in
the art. Cells such as *E. coli* are useful in this
15 respect.

 In another embodiment, the expression is transient
and may be mediated through means such as are known to
those skilled in the art, for example, by the
microinjection of mRNA molecules encoding one or more
20 glucose transport isoforms of interest. Other methods to
generate transient expression of a nucleic acid molecule
include, for example, ballistic methods, transient
transfection and electroporation.

 In a preferred embodiment, the mRNA corresponds to a
25 DNA sequence which is manipulated such that its coding
sequence is optimized for expression, according to the
codon usage preference tables for the organism in which
the mRNA is expressed. The mRNA can be isolated from a
biological source or generated by *in vitro* transcription
30 of a glucose transporter-encoding DNA, methods for both
of which are well understood by those skilled in the art.
Glucose transporter-encoding DNAs are known in the art.
A table of such sequences is provided below and each of
the sequences is readily available in public databases
35 such as GenBank.

Identified GLUT Sequences and Accession Numbers

<u>Transporter</u>	<u>Human</u>	<u>Mouse</u>	<u>Rat</u>	<u>Chicken</u>	<u>Pig</u>	<u>Trout</u>	<u>Cannis</u>	<u>Carp</u>	<u>Drosophila</u>	
GLUT1	NM006516	X69697	M13979	L07300	X17058	AF247728		AF247730	AF064703	448
	K03195	M23384						<u>Rabbit</u>	<u>Bovine</u>	
		M22998						M21747	M60448	
GLUT2	NM00340	X16986	NM_012879	Z22932		AF321816				
	XM003153	X15684	J03145							
	J03810									
GLUT3	NM006931	NM011401	NM_017102	M37785	L39214				L35267	
	XM006927	X61093	D13962							
	M20681	X69698								
		M75135								

GLUT4	NM001042	NM009204	NM_012751	AF247395
	XM008339	BB004644	D28561	
	M20747	AB008453	M25482	
			X14771	
			J04524	
GLUT5	NM003039	NM019741	D13871	
	XM001557			
	M55531			
GLUT8	NM04580	NM_019488	AJ245935	
	XM011828	AF232061	AB033418	
	Y17801	Y17802		
GLUT9	NM020041			
	XM003589			

	AF210317	
GLUT10	NM030777	
	NM030807	
GLUT11	NM017585	
	XM011837	

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5 In other embodiments, the expression of the glucose transporter isoforms of interest is accomplished through the use of genetic elements integrated into the organism's genome or into a stable extra chromosomal element. In these embodiments, it may be desirable to
10 "knock-out" any endogenous basal glucose transporters, such that the heterologous glucose transporter isoforms are the sole or substantially dominant source of facilitated glucose transport. This allows the straightforward measurement or determination of side-
15 effects of added protease inhibitors in the relative absence of background noise. Such "knock-out" mutants are well known in yeast and in other systems, and numerous strategies exist for the generation of such cell lines; combined with stable expression, they allow for
20 routine and standardized assays, and may offer certain advantages over transient expression methods.

 In another embodiment, the membrane system comprises membranes other than those in whole cells. Examples of membrane system for use with transmembrane proteins are
25 known to those skilled in the art. Typically such membrane systems comprise phospholipid or other bipolar lipids which provide both hydrophobic and hydrophilic properties. Examples of such systems include cell
30 membranes, cell ghosts, erythrocyte ghosts, membrane-derived vesicles, lipid-containing vesicles, artificial membranes, lipid-containing monolayers, black lipid membranes, reconstituted membranes, hybrid bilayer membranes, supported bilayer membranes, phospholipid-containing membranes or lipid-containing micelles.

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5 In one of its aspects, the method involves a detectable glucose. In one embodiment, the detectable glucose is isotopically labeled. Isotopes, such as those of carbon, iodine, fluorine, and hydrogen may be used for labeling the glucose molecules of the present invention. 10 Detection of such isotopically labeled glucose molecules is known in the art and is accomplished by measuring radioactive decay or mass differences, depending on the type of isotope used. Nuclear magnetic resonance, such as in vivo NMR can be used to measured isotopically- 15 labeled glucose.

 The glucose compound is typically a glucose or a glucose analog. Glucose analogs include, for example, methyl glucose, a-methyl glucose, 3-O-methyl glucose, deoxyglucose, 2-deoxyglucose, fluorodeoxyglucose, 2- 20 fluoro-2-deoxy-D -glucose, 3-fluoro-3-deoxy-D-glucose, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG), and other transportable glucose analogs. Transportable analogs are preferred for most applications, but nontransportable analogs may be used in 25 certain embodiments. The detectable analogs include radiolabeled molecules, those molecules detectable by emitting energy at specific wavelengths, those molecules detectable by absorbing energy at specific wavelengths, and molecules with detectable mass or atomic differences.

30 In another embodiment, the glucose is detected via interaction with soluble or immobilized enzyme, one or more substrates or resultant reactants of which are measured, for example electrochemically or optically. In other embodiments the glucose is detectable through other 35 means such as are known to those skilled in the art, for

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5 example, an appropriately "labeled" glucose compound may
be detectable through optical methods, such as
photometric means, fluorometric means, spectroscopic
means, or colorimetric means. Alternative means for
detecting a glucose compound include, but are not limited
10 to, isotopic means, electrochemical means, or immunologic
means. Infrared or near-infrared radiation spectroscopy,
impedance methods, including radiowave impedance, and
polarized light rotation are further methods which may be
used for measuring glucose compounds in specific
15 embodiments. Instruments for detecting molecules by such
means are well known in the art. For example, samples
may be placed in commercially available glucometer
systems, according to the manufacturer's instructions.
The foregoing are meant to be illustrative of, and not
20 limiting as to, the methods of detection.

In some embodiments the glucose compound molecule
may be a transportable analog or substituted molecule
wherein the detectability of the transportable molecular
entity may be provided by a nonglucose portion of
25 molecule, and detection is by any means known in the art,
such as, but not limited to those enumerated above.

Another aspect of the methods of the present
invention involves the use of protease inhibitors,
particularly antiretroviral protease inhibitors, for
30 screening for metabolic side-effects, or for use as
selective inhibitors of glucose transporter isoforms.
Such protease inhibitors may be approved or experimental
drugs, drug candidates, or lead compounds in drug
discovery efforts. Other embodiments include the targets
35 of rational drug design and/or the products of

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5 combinatorial chemistry directed at, for example,
antiretroviral drug development.

In one embodiment, the protease inhibitors are
antiviral and more specifically antiretroviral
therapeutic agents. In some embodiments the protease
10 inhibitors comprise therapeutic cocktails, mixtures or
combinations of antiviral compounds. In other
embodiments, the protease inhibitors are cruder extracts
in various stages of purification or characterization, or
pure compounds, such as, but not limited to:
15 peptidomimetic substrates; peptidomimetic substrate
analogs or derivatives; aspartyl protease inhibitors;
indazole- or other derivatives of cyclic ureas;
sulfonamides; derivatives of 2, 4-diamino-3-
hydroxycarboxylic acid; derivatives of phosphoric acid;
20 and/or the aspartyl proteinase inhibitors described in
U.S. Patent No. 5,945,413, and the like.

In one embodiment of the protease inhibitor
screening method, protease inhibitors are added to the
assays at various time points before during or after the
25 initiation of glucose transport measurement or before,
during or after insulin stimulation to determine the
kinetic properties of the protease inhibitor's effect on
glucose transport. It is well known in the art that
determining the kinetic properties of a molecular
30 interaction can lead to deeper understanding of the
mechanisms, which will ultimately lead to the
identification or development of compounds with improved
protease inhibitor effects and eliminated or optimized
ratio of protease inhibitor effect to metabolic side-
35 effects. Such kinetic assays are valuable in

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5 understanding the nature of the interaction of each
protease inhibitor tested with the glucose transport
apparatus.

10 The invention also provides for glucose transport
altering substances to be added to the assays. Glucose
transport altering substances include hormones, such as
15 insulin, synthetic hormones, and hormone analogs, as well
as other compounds, for example, peptides and drugs,
which are capable of altering glucose transport in cells.
Glucose transport altering substances may have their
15 action directly on the glucose transporters of the
invention, or where intact cells are used, the glucose
transport altering substances may operate through
biological signaling cascades and may involve secondary
message compounds directly or indirectly.

20 In another aspect of the present invention, a new
and useful method for the rational design of
antiretroviral protease inhibitors is provided. The
method comprises the following steps: 1) identifying a
drug candidate or lead compound; 2) testing the
25 compound to determine that it is effective in inhibiting
the aspartyl protease of the HIV or other retroviruses;
3) further testing the compound for inhibition in
glucose transport assays; 4) assessing the compound's
effects on glucose transport; and 5) selecting compounds
30 which are effective at inhibiting the aspartyl protease
in step 2) and possess minimal undesirable side-effects
in step 3). Most desirable are those compounds which
further satisfy all other clinical, toxicological and
pharmacological requirements for a new drug.

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5 One useful advantage of this method is that by
incorporating, from the earliest design stages, an assay
for the unwanted side-effects, expensive and time-
consuming efforts which lead to dead-end compounds can be
minimized. Since the particular undesirable metabolic
10 side-effects of the protease inhibitors are already
recognized as a substantial problem, this method of
rational design incorporating the strategy of testing
for a known metabolic side-effect early on in the design
process, will ultimately lead to more efficient drug
15 development programs for retroviral diseases.

It is anticipated that in various embodiments, such
a rational drug design method could include lead
compounds which can be detected or identified by a
variety of screening methods. One embodiment employs
20 high-throughput screening, which has proven useful for
identifying compounds with promise as drug candidates.
Another embodiment uses combinatorial chemistry, which
has also proven itself as a strategy for generating lead
compounds. Other strategies contemplated for use with
25 this invention include shotgun approaches and rational
screening programs. In another embodiment the lead
compounds are generated through ethnobotanical screening
programs and the like, whereby antiviral compounds from
plants and other natural sources are also contemplated as
30 sources of drug candidates for the method of the present
invention.

The testing of drug candidates for effects on
glucose transport employs the method as described above
in one embodiment. In another embodiment, such a method
35 may be simplified for screening purposes to measuring a

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5 specific molecular or physical interaction between a
membrane-free or membrane-bound glucose transporter and a
protease inhibitor. Such interactions between a
transmembrane transporter and an inhibitor are known in
the art. Analogous molecular interactions may occur
10 between enzymes and inhibitors. Some examples of these
interactions include covalent modification, hydrophobic
interaction, ionic interactions, zwitterionic or
amphiphillic interactions, hydrophillic interaction,
site-specific binding, occlusion of an active site, and
15 blocked access to a translocation channel. Although the
exact mechanism of the molecular interactions remain
uncertain, it is clear that these interactions occur and
that they are not nonspecific, being properties of
particular molecules and not others. In the present
20 invention, it has been shown that the antiretroviral
protease inhibitors selectively inhibit the Glut4 but not
the Glut1 isoforms of the glucose transporter.

The simplified method of quickly measuring a
molecular or physical interaction between the glucose
25 transporter and a protease inhibitor typically comprises
a glucose transporter in an artificial system.
Artificial systems such as lipid vesicles, micelles,
monolayers, or artificial membranes containing glucose
transporter molecules are contemplated herein. The
30 glucose transporter can also be solubilized in a manner
which allows interaction with the protease inhibitors to
occur. Solubilized transporters are known in the art, as
are methods for solubilizing membrane proteins, such as
glucose transporters.

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5 Such simplified assays allow miniaturization and
automation. These *in vitro* model assays can be conducted
as part of high throughput screening program, with the
more traditional cell-based glucose transport assay as a
follow-up. Detection of a potentially inhibitory
10 interaction between a glucose transporter and a protease
inhibitor in such an assay could be by any of the methods
well-known and widely-used in the art, for example by the
use of fluorescently-labeled glucose transporter and the
measurement of quenching of the fluorescent signal during
15 interaction with an appropriate inhibitor. Other methods
of measuring or detecting molecular interactions between
such molecules as membrane components and soluble
components are known in the art and may be used in
conjunction with the instant invention. For example,
20 either the glucose transporter or the protease inhibitor
can be labeled with a photolabile component which when
exposed to a particular wavelength light will result in a
chemical reaction, for example, the covalent binding of
portions of the interacting molecules which are within a
25 certain proximity. This covalent interaction allows the
specific portions of interacting molecules to be
identified, by various means, such as mass spectroscopy,
fluorescent detection, spectroscopic means, photometric
means, and separation means such as chromatographic
30 means. These covalently modified portions can then be
identified and the portions of the molecules which were
physically interacting can be deduced from the data.

Derivatization of inhibitors with fluorescent,
isotopic or photolabile labels can be used to provide a

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5 direct measurement of inhibitor binding to or interaction with the glucose transporter.

The glucose transporters of the molecular interaction method above are obtained from biological sources by extraction and or purification by means known
10 in the art. Other methods for obtaining glucose transporter molecules for measuring molecular interactions with protease inhibitors include *in vitro* translation from glucose transporter-encoding mRNAs, or combined *in vitro* transcription/translation from glucose
15 transporter-encoding DNA molecules. Synthetic glucose transporter molecules can be made from known amino acid sequences, or sequences altered therefrom, for use in the molecular interaction method. Additionally, in one embodiment, portions of glucose transporters may be
20 synthesized or generated from biologically derived glucose transporters for efforts to to determine particular molecular domains of the glucose transporters involved in molecular interaction with protease inhibitors. The glucose transporters obtained as
25 described herein can also be used in the cell-free methods of measuring glucose transport as described herein, including by incorporation into lipid vesicles, bilayers and the like.

In a preferred embodiment, the most promising drug
30 candidates for preclinical studies, as well as further toxicological, pharmacological and clinical studies are those compounds which are initially selected by a high through-put screening method for the properties of (1) protease inhibition and (2) no significant interaction
35 with glucose transporter, and optionally (3) which

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5 continue to show no significant effect on glucose transport by the transport method described above. Such drug candidates will certainly yield the most beneficial results throughout the further steps of approval as new drugs.

10 In a different aspect of the instant invention, screening assays are provided to identify target therapeutic compounds which can restore insulin-dependent glucose transport. For such assays, a model is used wherein a glucose transporter is produced by a cell. The
15 method comprises the steps of providing a reaction medium comprising cells that produce one or more glucose transporters, a quantity of an inhibitor of retroviral protease that reversibly inhibits insulin-dependent glucose transport of the transporter, a quantity of
20 insulin, and a glucose compound; measuring the amount of the glucose compound transported into the cells under preestablished conditions for a preestablished time period; adding the test compound to the reaction medium; measuring the amount of the glucose compound transported
25 into the cells under preestablished conditions for a preestablished time period; and determining the difference between the amount of glucose transported into the cells after addition of the test compound and the amount of glucose transported into the cells before
30 addition of the test compound, an increase in the amount of glucose transported into the cells after addition of the test compound being indicative that the test compound is capable of reversing the inhibition of the insulin-dependent glucose transport caused by the protease
35 inhibitor.

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5 The method provides for the screening of drugs which
act directly at the site of reversible insulin
resistance, i.e. compounds which specifically reverse-
insensitivity of the glucose transporter, particularly
the Glut4 isoform. Compounds which reverse the insulin
10 resistance may also work at other, secondary, locations
remote to the glucose transporter since these assays are
typically performed in intact cells. In a cell-free
mode, primarily compounds are detected in the screening
method which act more directly on the glucose
15 transporter. The method is particularly valuable as part
of a program of developing new drug candidates for
reversing insulin resistance. Following the screening
assay, candidate compounds are able to be tested in in
vivo models and then sent to preclinical and clinical
20 studies

 In one embodiment, the invention provides cells that
are specifically engineered to have a dominant glucose
transporter as an reversibly-inhibitable insulin-
sensitive glucose transporter. Such cells produce a
25 glucose transporter by expressing the gene product of a
nucleic acid molecule which encodes a glucose
transporter. In a preferred embodiment, the glucose
transporter is known to be susceptible to inhibition by
antiretroviral protease inhibitors. In one embodiment
30 the glucose transporter is a Glut4 isoform.

 In another embodiment, in vivo methods are provided
to assay target therapeutic compounds for the reversal of
protease inhibitor induced insulin-resistance using the
euglycemic-hyperinsulinemic clamp procedure. This method
35 is particularly useful for assaying compounds which have

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5 been promising in the in vitro methods as described
above.

10 The following examples are provided to describe the
invention in greater detail; they are intended to
illustrate, not to limit, the invention.

Example I.

15 The Mechanism of Insulin Resistance Caused by HIV
Protease Inhibitor Therapy

Methods:

Materials.

20 Indinavir, ritonavir, and amprenavir were obtained
from Merck, Abbott, and Glaxo Wellcome, respectively.
Xenopus laevis imported African frogs were purchased from
Xenopus Express (Homasassa, FL). All other reagents
unless otherwise specified were obtained from Sigma.

25 Cell culture of 3T3-L1 adipocytes.

3T3-L1 fibroblasts obtained from the American Type
Culture Collection were grown to confluence and 48 h
later subjected to the differentiation protocol described
previously (Tordjman et al. 1989). Mature 3T3-L1
30 adipocytes were maintained in DMEM supplemented with 10%
fetal bovine serum and used 10 to 15 d post-
differentiation.

2-Deoxyglucose uptake measurements in 3T3-L1 adipocytes.

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5 3T3-L1 adipocytes grown in 3.5 cm dishes were serum-starved for at least 3 hours and then washed three times with Krebs-Ringer phosphate buffer. [³H]-2-deoxyglucose uptake (50 μ M cold 2-deoxyglucose) was measured in Krebs-Ringer phosphate buffer as described previously (Tordjman et al. 1989) for 6 min at 37 °C under basal and insulin-stimulated conditions (1 μ M insulin for 20 min). Where so indicated, HIV protease inhibitors (indinavir, ritonavir, or amprenavir) were added to the cells at designated concentrations 6 minutes prior to the assay. 10 Stock solutions of indinavir and amprenavir were made in water. Ritonavir was dissolved in ethanol. When adding ritonavir to cells, the final concentration of ethanol was less than 0.5%. Non-specific uptake was measured in the presence of 20 μ M cytochalasin B and subtracted from 15 the experimental values. 20

Subcellular fractionation of 3T3-L1 adipocytes.

 3T3-L1 adipocytes were grown in 10 cm² dishes and incubated at 37 °C for 4 hours in serum-free DMEM in the 25 absence or presence of 100 μ M indinavir. After treatment with or without insulin (1 μ M for 20 min), the cells were scraped in ice-cold HES buffer (20 mM HEPES, pH 7.4, 255 mM sucrose, and 1 mM EDTA) supplemented with 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium 30 vanadate, and general protease inhibitors (1 μ g/ml leupeptin, 1 μ g/ml antipain, 5 μ g/ml trypsin inhibitor, 1 μ g/ml chymostatin, 1 μ g/ml pepstatin A, and 0.5 mM phenylmethylsulfonyl fluoride). After homogenization through 11 passes in a Yamato LSC homogenizer (1200 rpm) 35 at 4 °C, subcellular fractionation by differential

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5 centrifugation was performed as described previously
(Piper et al., 1991).

Immunoblot analysis.

10 3T3-L1 adipocyte fractions were subjected to SDS-
PAGE and transferred to nitrocellulose. Glut1 and Glut4
transporters were detected using polyclonal antibodies
raised against peptides corresponding to the carboxy-
terminal 16 residues of the respective transporter
isoform. The autoradiographic signals were quantified by
15 using a phosphorimager (Molecular Dynamics).
Phosphotyrosine-containing proteins were detected using
the monoclonal PY-20 antibody (Transduction
Laboratories). Phospho-Akt specific antibodies (New
England Biolabs) were used to detect Akt phosphorylated
20 at threonine 308 and serine 473.

Confocal immunofluorescence microscopy.

25 3T3-L1 adipocytes were grown on No. 1 glass
coverslips. Cells were incubated in the absence or
presence of 100 μ M indinavir as described above for
subcellular fractionation. After treatment with or
without insulin (1 μ M for 20 minutes), whole cells were
fixed immediately in 4% paraformaldehyde and
permeabilized using methanol. PM sheets adherent to the
30 coverslip were prepared by gentle sonication as described
previously (Robinson et al., 1992) and subsequently fixed
using 4% paraformaldehyde. Glut1 and Glut4 subcellular
distributions in the prepared coverslips were visualized
by indirect immunofluorescence microscopy using isoform-
35 specific polyclonal antibodies essentially as described

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5 previously (Robinson et al., *supra*). Images were taken using a Bio-Rad MRC-1024 laser scanning confocal microscope.

2-Deoxyglucose uptake measurements in Xenopus oocytes.

10 *Xenopus laevis* oocytes were prepared and injected as described previously (Keller et al., 1989) with 50 ng of either Glut1 or Glut4 mRNA synthesized *in vitro* (Megascript RNA synthesis kit, Ambion). After a three day incubation in Barth's saline containing albumin at 18
15 °C, groups of 15-20 oocytes were washed and [³H]-2-deoxyglucose (50 µM) uptake measurements were performed in Barth's saline at 22 °C for 30 minutes. HIV protease inhibitors (indinavir, amprenavir, or ritonavir) were added to the assay mixture immediately prior to the
20 uptake measurement.

Results:

2-Deoxyglucose uptake measurements in 3T3-L1 adipocytes.

When 3T3-L1 adipocytes were treated with indinavir,
25 a statistically significant dose-dependent decrease in insulin-stimulated glucose uptake was inhibited 63% at the maximum concentration of indinavir tested (100 µM; Fig. 1A). At 10 µM, indinavir inhibited insulin-stimulated glucose uptake by 26% ($p < 0.0001$). Basal
30 glucose uptake was largely unaffected by indinavir, although at 20 µM indinavir, a modest increase was reproducibly observed. The inhibitory effect of indinavir on insulin-stimulated glucose uptake was very rapid, as the drug was added to the cells only 6 minutes
35 prior to the uptake assay. Furthermore, removal of

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5 indinavir rapidly restored normal insulin-responsive
glucose uptake within 30 minutes (data not shown).
Amprenavir and ritonavir, also exhibited an effect
comparable to that of indinavir (Fig 1B).

10 *Immunoblot analysis of 3T3-L1 adipocyte fractions.*

Immunoblot analysis of 3T3-L1 adipocyte subcellular
fractions with anti-phosphotyrosine antibodies revealed
that insulin receptor (IR) autophosphorylation and
subsequent tyrosine phosphorylation of insulin receptor
15 substrate-1 (IRS-1) occurred normally in cells exposed to
indinavir (Fig. 2A). The *in vivo* phosphorylation status
of the downstream Akt kinase was assessed using phospho-
Akt specific antibodies.

Indinavir had no effect on the insulin-stimulated
20 phosphorylation of Akt on threonine 308 or serine 473
(Fig. 2B). The glucose transporter content in the plasma
membrane (PM) fractions detected by isoform-specific
antibodies increased with insulin by 81% and 63% for
Glut1, and by 36% and 38% for Glut4 in control and
25 indinavir-treated cells, respectively. Concomitantly,
the transporter content in the low density microsome
(LDM) fractions decreased by 37% and 48% for Glut1, and
by 21% and 19% for Glut4 in control and indinavir-treated
cells, respectively (Fig. 2C).

30 *Confocal immunofluorescence microscopy.*

Both control and indinavir-treated cells exhibited
increased Glut1 and Glut4 staining at the plasma membrane
upon stimulation with insulin. The subcellular

- 40 -

5 distribution of glucose transporters was unchanged in
indinavir-treated samples relative to control cells.

2-Deoxyglucose uptake measurements in Xenopus oocytes.

Indinavir had no effect on Glut1 activity in *Xenopus*
10 oocytes. Remarkably, however, the activity of Glut4
expressed in oocytes was inhibited by 45% at the maximum
dose of indinavir tested (100 μ M), an effect of
comparable magnitude to that observed in insulin-
stimulated 3T3-L1 adipocytes (Fig. 3A). Amprenavir and
15 ritonavir also selectively inhibited Glut4 by 54% and
42%, respectively (Fig. 3B).

Discussion:

The effects of the HIV-1 protease inhibitor,
20 indinavir, on glucose transport in 3T3-L1 adipocytes, a
system that responds robustly to insulin, were initially
examined. At 10 μ M, which is within the physiologic
range of plasma concentrations achieved in vivo in HIV
patients, indinavir inhibited insulin-stimulated glucose
25 uptake by 26% ($p < 0.0001$). Inhibition of insulin-
stimulated glucose uptake appears to be a general
property of HIV-1 protease inhibitors, as two other
compounds within this class, amprenavir and ritonavir,
also exhibited inhibitory effects comparable to that of
30 indinavir. As the metabolic effects of insulin require
PI-3 kinase activation, the *in vivo* phosphorylation
status of the downstream Akt kinase was assessed using
phospho-Akt specific antibodies. Indinavir was found to
have no effect on the insulin-stimulated phosphorylation
35 of Akt, thus demonstrating that the PI-3 kinase signaling

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5 pathway remained intact. Insulin acutely stimulates
glucose uptake in muscle and fat cells by triggering the
translocation of intracellularly sequestered glucose
transporters, predominantly the Glut4 transporter
10 isoform, to the plasma membrane. 3T3-L1 adipocytes
express Glut1 and Glut4, and both of these transporter
isoforms appeared to translocate properly to the cell
surface in response to insulin despite the presence of
100 μ M indinavir. Confocal immunofluorescence microscopy
15 of whole cells and plasma membrane A sheets also showed
that the subcellular distribution of glucose transporters
was unchanged in indinavir-treated samples relative to
control cells. The rapid onset of inhibition observed in
the glucose uptake assay (Fig. 1), in which indinavir was
20 added to the cells after sufficient time had elapsed for
the majority of the transporters to reach the plasma
membrane following insulin stimulation, is consistent
with indinavir acting at a site subsequent to the
translocation of transporters to the plasma membrane.
Additionally, the extent of inhibition of transport
25 activity did not change if indinavir was added either
before or after 20 min of insulin stimulation (data not
shown).

Glut1 and Glut4 were heterologously expressed in
Xenopus laevis oocytes by microinjection of their
30 respective mRNA in order to test the possibility that
indinavir might be directly inhibiting the intrinsic
transport activity of glucose transporters. Glut1
activity was unaffected, however the transport activity
of the Glut4 isoform was substantially inhibited by all
35 three protease inhibitors tested. The data obtained in

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5 *Xenopus* oocytes are consistent with what is observed in
3T3-L1 adipocytes, in which basal (indinavir-resistant)
and insulin-stimulated (indinavir-inhibitable) glucose
uptake are largely mediated by Glut1 and Glut4,
respectively. From the data presented, it is concluded
10 that HIV protease inhibitors unexpectedly act as potent,
isoform-specific inhibitors of the transport function of
the Glut4 glucose transporter.

 This is the first demonstration that pharmacologic
manipulation of glucose transport is feasible in a
15 selective manner. An agent that can reversibly induce an
insulin resistant state would be a very useful tool in
developing model systems that mimic type 2 diabetes.
Glut4 is predominantly expressed in tissues responsible
for the bulk of whole body glucose disposal
20 (skeletal/cardiac muscle and fat) and is believed to be
the principal transporter isoform mediating insulin-
stimulated glucose uptake at these sites. As glucose
transport is the rate-limiting step for whole body
glucose disposal in rodents and in humans, the inhibitory
25 effect of antiretroviral protease inhibitors on Glut4 is
therefore likely to be the direct cause of insulin
resistance observed in HIV patients receiving this class
of drugs.

 In predisposed individuals, diabetes can result
30 after pancreatic b cells fail to compensate for the
insulin resistance. A recent clinical study employing a
longitudinal design comparing fasting glucose and insulin
levels before and after administration of protease
inhibitor therapy demonstrated that insulin resistance is
35 apparent after a relatively short period of time (an

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5 average of 3-4 months between measurements) before
significant changes in body weight and fat distribution
occur.

The fact that insulin resistance appears to precede
the manifestation of lipodystrophy is consistent with our
10 hypothesis that indinavir directly causes insulin
resistance through its effect on Glut4, rather than
insulin resistance developing secondarily to the
lipodystrophy.

It is possible that insulin resistance occurs much
15 earlier than reported thus far, perhaps even immediately
upon initiation of protease inhibitor therapy. Moreover,
if this hypothesis is correct, insulin resistance should
be maximal when *in vivo* protease inhibitor concentrations
are maximal. Thus, depending on the dosing regimen and
20 the pharmacokinetic characteristic of the protease
inhibitor used, simple measurements of fasting glucose
and insulin levels may be underestimating the true extent
of insulin resistance that actually occurs.

A 'knockout' mouse that lacks Glut4 is insulin
25 resistant, and interestingly, almost devoid of fat
tissue. Thus, Glut4 activity *per se* may somehow be
required for adipogenesis. If this is true, the protease
inhibitor's direct effect on Glut4 may account for the
clinically observed lipodystrophy in addition to the
30 insulin resistance.

Recent reports that HIV protease inhibitors
interfere with adipogenesis in cultured cell models do
not contradict this hypothesis. HIV patients treated
with protease inhibitors show a characteristic loss of
35 adipose tissues at peripheral sites as opposed to the

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5 abdomen. One can speculate that peripheral adipocytes preferentially synthesize lipid de novo from blood glucose, whereas abdominal adipocytes may obtain their lipid primarily from circulating triglycerides.

10 As antiretroviral protease inhibitors play a vital role in prolonging the life span of HIV patients and are often administered over an extended period of time, the metabolic side effects and their chronic or acute consequences are likely to be more prevalent in the future. Further drug development is necessary in order
15 to design new compounds that maintain the efficacy in the management of HIV infection, but that also minimize the detrimental effect on the glucose transport system observed in this study.

20 Example 2.

In vivo Verification of
the Mechanism of Insulin Resistance
Caused by HIV Protease Inhibitor Therapy
25 and Application of Same.

Materials and Methods:

Male Wistar rats (Charles River Corp) weighing between 250 - 400 g were used for all experiments
30 described below. Glucose measurements were made using a Glucometer Elite Glucometer (Bayer Corporation, Diagnostics Division, Tarrytown, NY). Protease inhibitor used was Indinavir (Merck). Jugular vein and carotid artery catheters were fabricated using microrenathane
35 tubing (Braintree Scientific) for venous catheters and

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5 PE50 tubing (Becton Dickenson) for arterial catheters. Catheters were inserted surgically under methohexital (Jones Pharma Inc, St. Louis, MO) anesthesia. Humulin R insulin (Eli Lilly, Indianapolis, IN) was used where indicated.

10 **Effect of Acute Indinavir Administration on Glucose Tolerance in vivo.**

To determine whether protease inhibitors acutely affect insulin sensitivity *in vivo*, the following
15 experiments was conducted: Following a 12 hour fast, male Wistar rats were given a single intraperitoneal injection of 50% dextrose, in water, to a dose of 2 g/kg, together with either Indinavir (10 mg/kg) or water (for Control animals). Plasma glucose levels were measured in
20 peripheral venous blood at times (t) = 0, 15, 30, 60, 90, and 120 min after injection. The results from 8 - 9 animals were averaged for each data point. Results are shown in FIG 4a. By t = 30 min, blood glucose concentrations were significantly elevated ($p < 0.05$) in
25 indinavir-treated animals (241 ± 11 mg/dl) relative to those of control animals (195 ± 15 mg/dl).

To determine even earlier effects of indinavir on *in vivo* glucose tolerance, at least 4 days prior to each experiment catheters were inserted, under methohexital
30 anesthesia, into the jugular veins of 200-300 gram male Wistar rats. Indinavir (15 mg/kg) was injected intravenously 5 minutes prior to the intraperitoneal injection of glucose (2 gm/kg). Blood was withdrawn via the venous catheter at t = 0, 2, 5, 10, 15, 30, 60, 90
35 and 120 min after glucose injection and plasma glucose

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5 concentrations were determined. Results are shown in
Figure 4b. Under these conditions, an even greater
elevation in plasma glucose concentrations was observed
following indinavir treatment. Peak glucose
concentrations were 407 ± 25 mg/dL in indinavir-treated
10 animals compared to 311 ± 30 mg/dL in water-treated
controls.

Example 3:

15 **Effect of Acute Intravenous Administration of Indinavir
on Peripheral Insulin Sensitivity *in vivo*.**

Peripheral insulin sensitivity in rats was assessed
by the euglycemic hyperinsulinemic clamp technique.
Venous and arterial catheters were surgically inserted
20 into the animals under anesthesia. At least 4 d prior to
the initiation of experimental treatments catheters were
inserted into both jugular vein and carotid artery
locations. Following a 12 hour fast, animals were
infused with insulin (40 mU/kg/min) and 50% dextrose in
25 water through the venous catheter. Blood was sampled
every 5 - 10 min through the arterial catheter for serum
glucose determinations. The rate of glucose infusion
(Glucose Infusion Rate, GIR) was adjusted as necessary to
maintain serum glucose at 100 - 110 mg/dl (FIG. 5A, 5B,
30 5C).

After obtaining a stable GIR (120 min), a water
infusion containing indinavir at 0.0, 0.3, or 0.5
mg/kg/min was started through the venous catheter. The
indinavir-containing infusion was discontinued after

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5 approximately 120 minutes and the euglycemic infusion was continued for approximately another 4 h.

A 50% decrease in the GIR required to maintain euglycemia was observed within 90 minutes after starting the intravenous infusion of indinavir at a rate of 0.5
10 mg/kg/min (FIG. 5C). At an indinavir infusion rate of 0.3 mg/kg/min, a 20% decrease in GIR was observed (FIG. 5B).

The reduction in the GIR required to maintain euglycemia reflects a decrease in the rate at which
15 glucose was being removed from the serum, thereby reducing the need for incoming glucose to maintain a homeostatic concentration of serum glucose. Insulin sensitivity was restored to baseline within 4 h after stopping the indinavir infusion, as reflected by the
20 increase in GIR (FIG. 5A, 5B, 5C)

In a separate set of experiments, the infusion of indinavir or water was started 30 minutes prior to the start of euglycemic hyperinsulinemic clamp procedure. Insulin sensitivity was assessed by the average glucose
25 infusion rate required to maintain euglycemia after equilibrium had been established. Statistically significant reductions in peripheral insulin sensitivity ($p < 0.05$) were observed both with an indinavir infusion rate of 0.3 mg/kg/min (15% reduction) and 0.5 mg/kg/min
30 (40% reduction) compared with controls (FIG. 6).

These data demonstrate that antiretroviral protease inhibitors cause acute and reversible changes in whole
body glucose homeostasis and selective inhibition of
35 GLUT4 glucose transporter isoform. The data also support

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5 the contribution of the GLUT4 isoform inhibition to the
development of insulin resistance in patients treated
with protease inhibitors.

10 The present invention is not limited in any manner
to the embodiments described and exemplified above. It
is capable of variation and modification in accordance
with the scope of the appended claims.

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5 We claim:

1. A method for screening an antiretroviral protease inhibitor for a metabolic side-effect on glucose transport, the method comprising the steps of:

- 10 a) providing a reaction medium comprising one or more glucose transporters in a membrane system through which the glucose transporters transport a glucose compound;
- b) adding to the reaction medium a detectable form
15 of the glucose compound;
- c) adding to the reaction medium the protease inhibitor to be screened;
- d) determining activity of the glucose transporter by measuring an amount of the glucose compound
20 transported by the glucose transporter; and
- e) quantifying the metabolic side-effects of the protease inhibitor screened as a function of the activity of the glucose transporter in the presence the protease inhibitor as compared with the activity of the glucose
25 transporter in the absence of the protease inhibitor.

2. The method of claim 1 wherein the glucose transporter is a Glut4 isoform.

30 3. The method of claim 1 wherein the membrane system is selected from the group consisting of cells, cell membranes, cell ghosts, erythrocyte ghosts, membrane-derived vesicles, lipid-containing vesicles, artificial membranes, lipid-containing monolayers, black lipid
35 membranes, reconstituted membranes, hybrid bilayer

- 50 -

5 membranes, supported bilayer membranes, phospholipid-containing membranes and lipid-containing micelles.

4. The method of claim 3 wherein the membrane system
10 comprises cells and the cells are selected from the group consisting of adipocytes, oocytes, kidney cells, cardiac cells, skeletal muscle cells, liver cells, neuronal cells, brain cells, *Xenopus* cells, *Escherichia coli*, *Saccharomyces cerevisiae*, insect cells, mouse cells, rat cells, chimpanzee cells, human cells, tumor cells,
15 cancerous cells, transformed cells, and genetically engineered cells.

5. The method of claim 3, wherein the membrane system
20 comprises cells and the cells contain one or more expressible nucleic acid molecules encoding the one or more glucose transporters.

6. The method of claim 5, wherein the nucleic acid
25 molecule encoding the glucose transporter is a heterologous nucleic acid molecule to the cells.

7. The method of claim 5 wherein the cells have a
30 substantially negligible basal level of glucose transport other than the glucose transport provided by the heterologous glucose transporter.

8. The method of claim 5 wherein the expression of the
35 glucose transporter is a stable characteristic of the cell.

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- 5 9. The method of claim 8 wherein the nucleic acid encoding the glucose transporter is integrated into the chromosome or is contained within an extrachromosomal element.
- 10 10. The method of claim 5 wherein the expression of the glucose transporter is a transient characteristic of the cell.
- 15 11. The method of claim 10 wherein the nucleic acid is an mRNA which is microinjected into the cell.
12. The method of claim 1, which further comprises a step of adding a glucose transport-altering substance to the reaction medium.
- 20 13. The method of claim 12 wherein the membrane system comprises cells and the glucose transport altering substance has the biological activity of a hormone.
- 25 14. The method of claim 13 wherein the hormone is insulin.
15. The method of claim 1 wherein the protease inhibitor is added to the reaction medium at any time during the
- 30 assay.
16. A kit for use in screening protease inhibitors for metabolic side effects on glucose transport, the kit comprising one or more components selected from the group
- 35 consisting of a reaction vessel, a cell line capable of

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5 expressing one or more glucose transporters, a membrane
system containing one or more glucose transporters, one
or more nucleic acid sequences encoding a glucose
transporter, one or more mRNA sequences encoding a
glucose transporter for transient expression, a
10 detectable glucose compound, a standardized protease
inhibitor, a control glucose transport inhibitor, glucose
transport altering substances, control cells, standards
for validating the assay, detailed instructions, quality
control certifications, disposable labware, and
15 disposable personnel protective items.

17. A method of developing improved therapeutic
compounds safe and effective in managing an infection
with a retrovirus, the method comprising the steps of:

20

a) identifying a therapeutic compound of interest;
b) testing the compound with the following steps;
1) determining that the compound inhibits the
aspartyl protease of the retrovirus;

25

2) using the compound as a protease inhibitor
in the method of claim 1; and

3) assessing the compound for inhibition of
glucose transport; and

30

c) selecting compounds which inhibit the aspartyl
protease and which do not inhibit glucose transport.

18. A cell-free assay for determining if a selected
glucose transporter physically associates with a selected
protease inhibitor, the method comprising the steps of:

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5 a) labeling the glucose transporter or the protease inhibitor, or both, with a detectable label, wherein a measurable feature of the detectable label changes if the glucose transporter physically interacts with the protease inhibitor;

10 b) adding the glucose transporter and the protease inhibitor to a reaction medium under conditions enabling physical association, if any, between the glucose transporter and the protease inhibitor; and

15 c) measuring a change, if any, in the measurable feature of the detectable label, the change being indicative that the glucose transporter and the protease inhibitor physically interact.

19. The assay of claim 18, wherein the glucose
20 transporter is a Glut4 isoform.

20. The assay of claim 18, wherein the physical interaction is selected from the group consisting of hydrophobic interactions, hydrophilic interactions,
25 covalent interactions, Van der Waal's interactions, ionic interactions.

21. The assay of claim 20, wherein the physical interaction results in occlusion of an active site of the glucose transporter or protease inhibitor or blockage of
30 the glucose transporter's glucose translocation channel.

22. The assay of claim 18 wherein the detectable label is selected from the group consisting of isotopic labels,
35 fluorescent labels, and photolabile labels.

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5

23. The assay of claims 18 wherein the measurable change in the detectable label is quenching of a signal produced by the detectable label.

10

24. The assay of claim 18 wherein the measurable change in the detectable label is a change in a spectrophotometric feature of the detectable label.

15

25. The assay of claim 18 wherein the glucose transporter is soluble in the reaction medium.

26. The assay of claim 18 wherein the glucose transporter is contained within a membrane system.

20

27. A method for screening therapeutic test compounds for their ability to alleviate insulin resistance, the method comprising the steps of:

a) providing a reaction medium comprising:

25

- i) cells that produce one or more glucose transporters;
- ii) a quantity of an inhibitor of retroviral protease that reversibly inhibits insulin-dependent glucose transport of the transporter;
- iii) a quantity of insulin; and
- iv) a glucose compound;

30

b) measuring the amount of the glucose compound transported into the cells under preestablished conditions for a preestablished time period;

c) adding the test compound to the reaction medium;

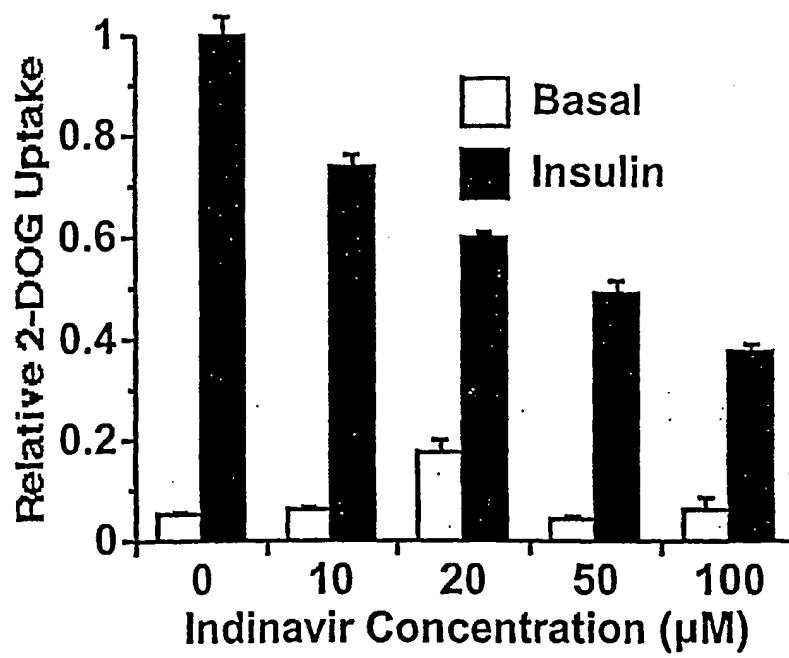
- 55 -

- 5 d) measuring the amount of the glucose compound
transported into the cells under preestablished
conditions for a preestablished time period; and
- 10 e) determining the difference between the amount of
glucose transported into the cells after addition of the
test compound and the amount of glucose transported into
the cells before addition of the test compound, an
increase in the amount of glucose transported into the
cells after addition of the test compound being
indicative that the test compound is capable of reversing
15 the inhibition of the insulin-dependent glucose transport
caused by the protease inhibitor.

28. The method of claim 27 wherein the quantity of
insulin is substituted with a quantity of a glucose
20 transport-altering substance.

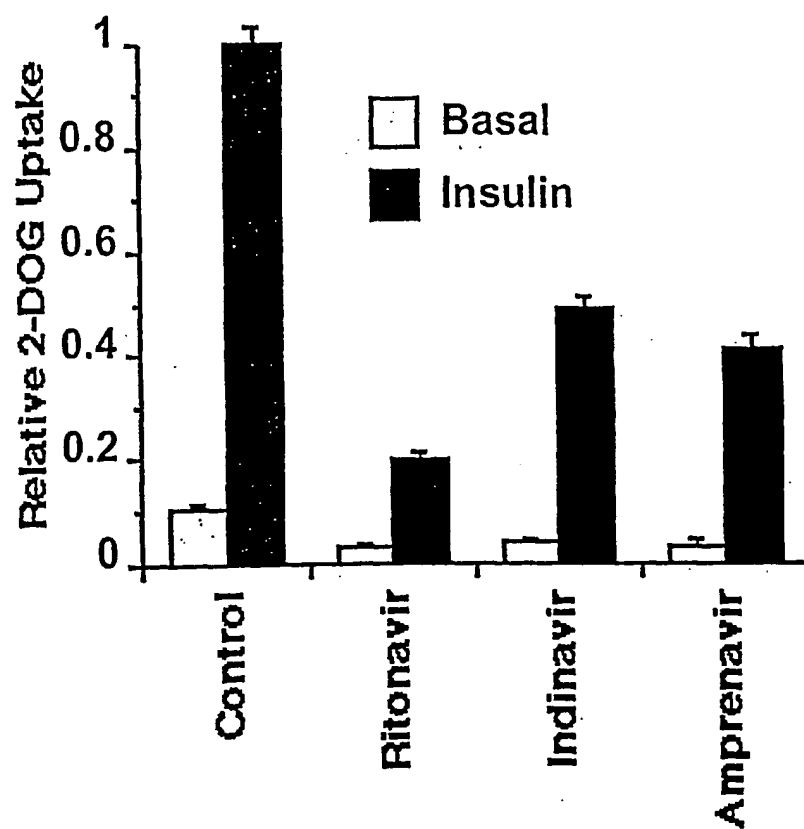
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Figure 1A



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Figure 1B



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Figure 2A

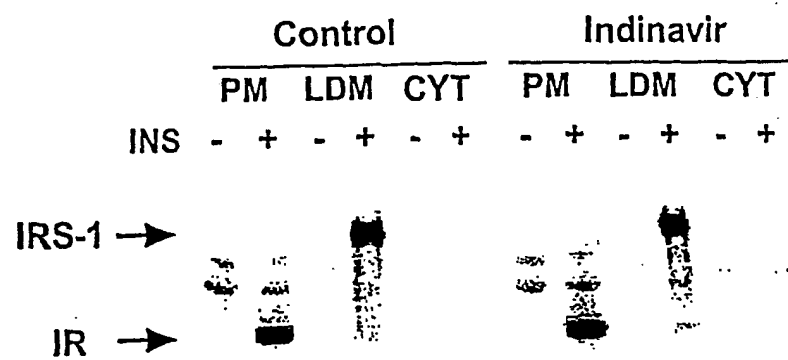
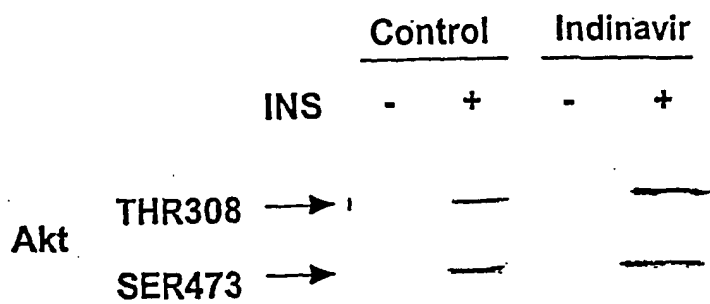
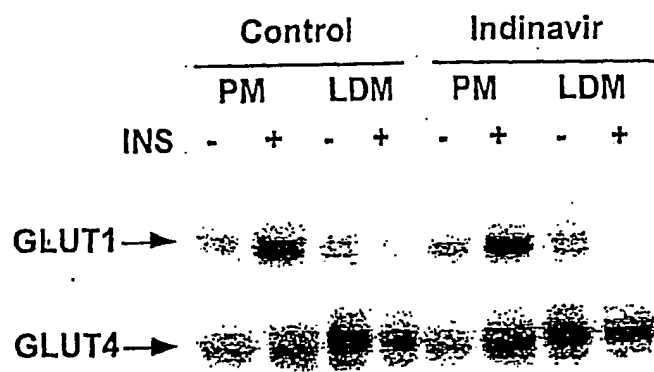


Figure 2B



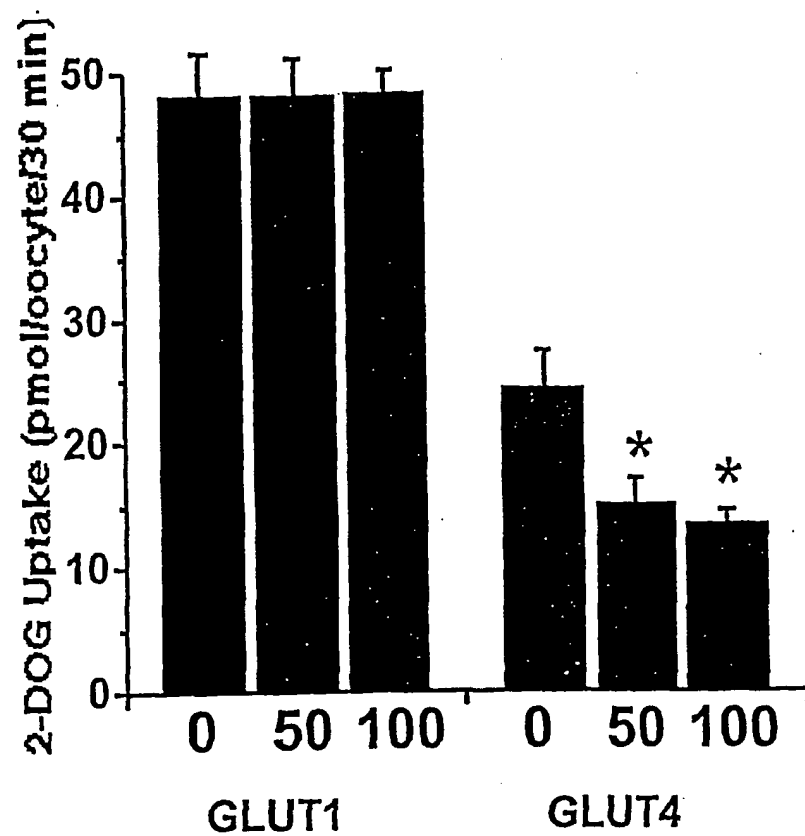
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Figure 2c



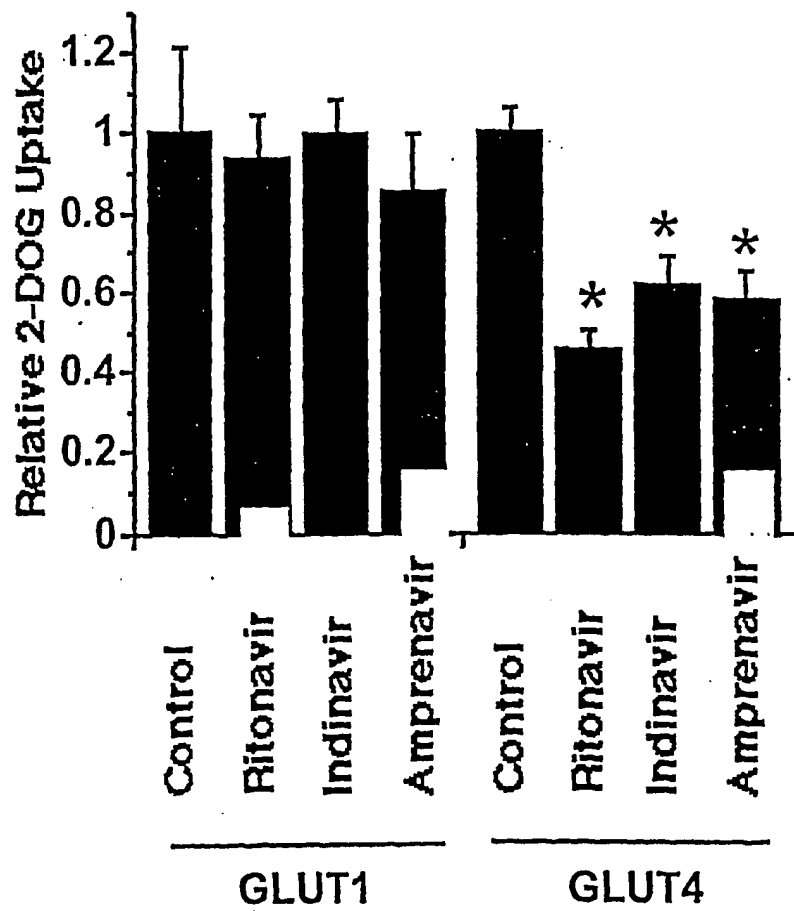
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Figure 3a



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Figure 3b



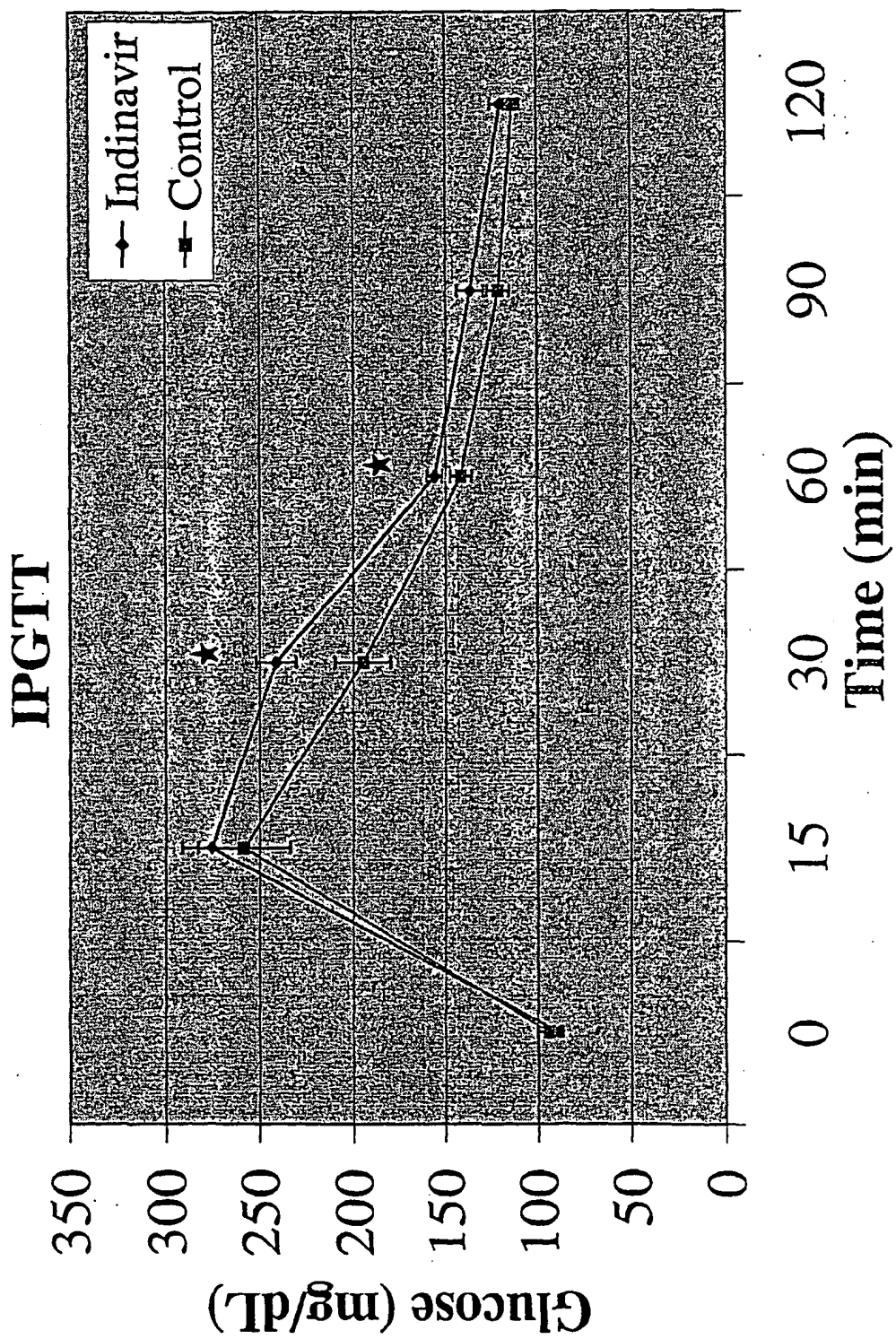


Figure 4a

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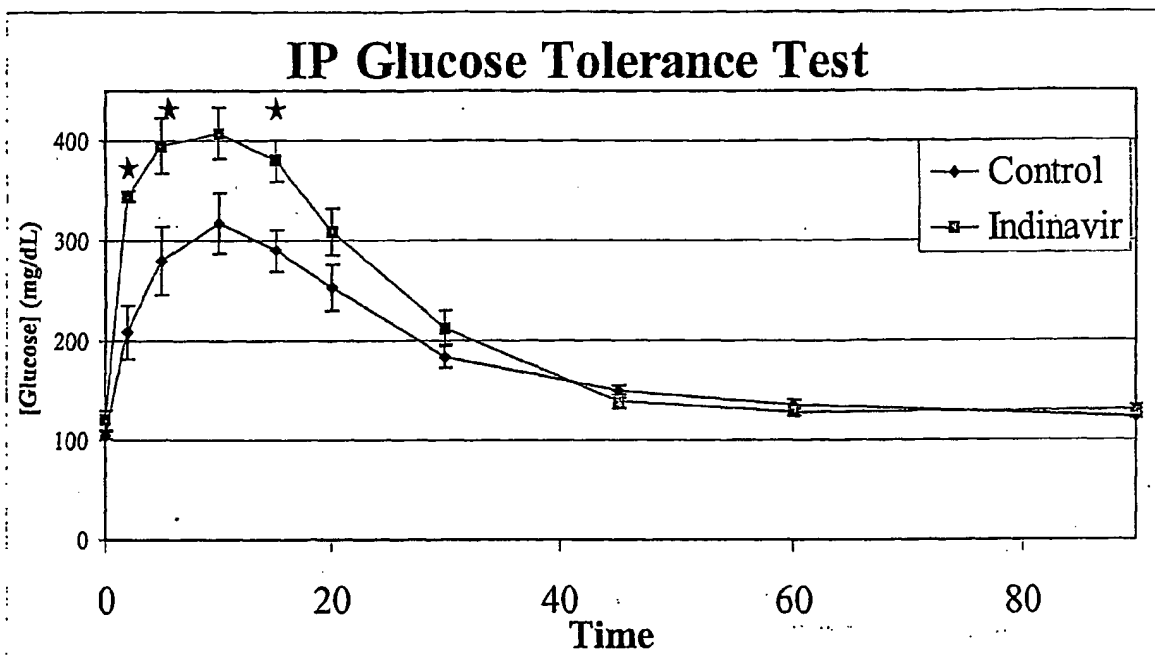
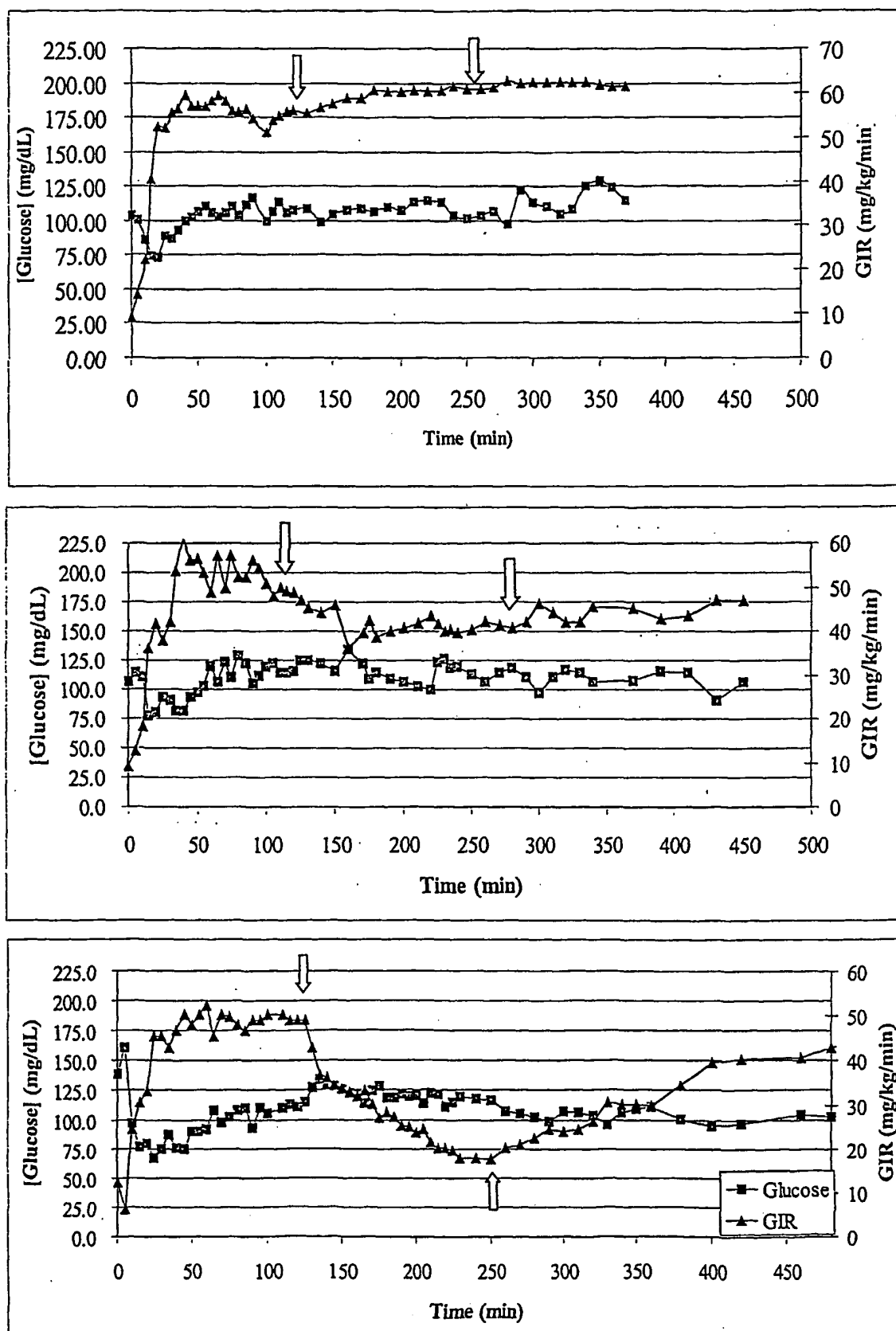


Figure 4b

Figure 5

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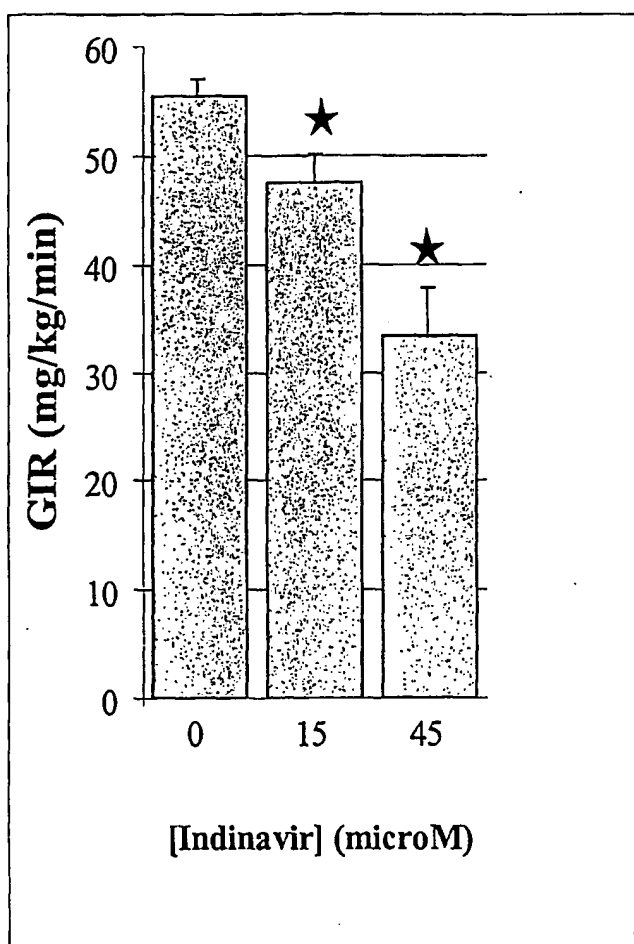
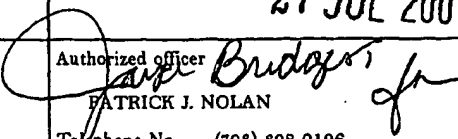


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/12697

A. CLASSIFICATION OF SUBJECT MATTER IPC(7) : G01N 33/567, 33/53; C12Q 1/02 US CL : 435/7.21, 29, 975 According to International Patent Classification (IPC) or to both national classification and IPC																				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/7.21, 29, 975 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) DIALOG EMBASE LIFESCI MEDLINE BIOSIS WEST																				
C. DOCUMENTS CONSIDERED TO BE RELEVANT																				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																		
Y,P	MURATA et al. The Mechanism of Insulin Resistance Caused by HIV Protease Inhibitor Therapy. J. Biol. Chem. 07 July 2000, Vol. 275, No. 27, pages 20251-20254, see entire document.	1-28																		
Y	DALE et al. 'Effects of Protease Inhibitors on Glucose Transporter Structure and Function in Bovine Cardiac Sarcolemmal Vesicles'. In: An annual meeting of Research Scientist: Experimental Biology 96 TM , (Washington, DC). FASEB Journal. 14-17 April 1996, Vol. 10, No. 3, Abstract No. 3824, see entire document.	1-28																		
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.																				
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"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																		
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"P" document published prior to the international filing date but later than the priority date claimed																				
Date of the actual completion of the international search 28 JUNE 2001		Date of mailing of the international search report 27 JUL 2001																		
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer  PATRICK J. NOLAN Telephone No. (703) 308-0166																		